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Three-dimensional composite of demineralized bone powder and collagen for *in vitro* analysis of chondroinduction of human dermal fibroblasts

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Implantation of demineralized bone powder (DBP) in muscle or connective tissue stimulates chondrogenesis followed by ectopic bone formation, in this way inducing the differentiation of endochondral bone. A new 3-dimensional *in vitro* composite sponge was designed to duplicate the packing density of *in vivo* DBP implants. The composite device, which consists of DBP packed between two layers of a porous collagen lattice, was used to assess the chondroblastic differentiation of human dermal fibroblasts. Important design considerations for this device were biocompatibility, rigidity and ability of cells to penetrate. In this study, collagen concentration and source, irradiation, and lyophilization conditions were varied in fabrication. Human dermal fibroblasts were seeded onto the composite sponge, migrated through the collagen lattice into the packet of DBP, and deposited a metachromatic extracellular matrix amongst the particles of DBP. In contrast, cells cultured in collagen sponges or in composite sponges with inactivated guanidine-extracted DBP did not secrete metachromatic matrix. This new *in vitro* system will be valuable in defining the mechanism of differentiation by osteoinductive materials and in evaluating the influence of other extracellular components and soluble factors on skeletal differentiation. © 1996 Elsevier Science Limited.

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Demineralized bone matrix induces the formation of cartilage and bone in soft tissue sites. Animal models have been used for the investigation of tissue responses to particles of demineralized bone matrix implanted intra-muscularly[1], subcutaneously[2] and intra-osseously[3]. These *in vivo* models have provided information on the regulation of chondro- and osteoinduction, but have not been useful for revealing the molecular events that mediate the differentiation of chondroblasts.

Several investigators have designed *in vitro* models to examine the early events in induced chondrogenesis. The first models by Urist and Nogami used hemicylinders of demineralized bone as a morphogenic substratum[4]. Embryonic cells or embryonic tissue outgrowths that were cultured on these hemicylinders generated foci of chondrogenesis, especially in available voids and cracks in the interior of the bone. In subsequent experiments, cartilage was reproducibly found within crevices that were cut into segments of demineralized bone[5,6]. This geometry suggests the importance of surface interactions between the inductive matrix and target cells. From those studies, it was not clear whether chondroinduction was a reflection of the pluripotentiality of the embryonic cells that were used. Instead of embryonic cells and tissues, Katho and Urist[7] used muscle explants from 21-day-old rats as the target cells. Although those cultures may not have the pluripotentiality of embryonic cells, it was difficult to identify the target cells in such heterogeneous systems. In order to model more closely the process of endochondral osteogenesis that is induced by subcutaneous implantation of DBP, we chose to test the *in vitro* effects of demineralized bone on more mature cells that do not have the chondrogenic potential displayed by many embryonic and neonatal cells, including adult human dermal fibroblasts[8].

Geometry and cell/substrate relationships appear to be important in evaluating induction *in vitro*. As shown by the localization of chondrogenesis within crevices and internal voids in inductive demineralized

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bone in Urist's *in vitro* studies, the 3-dimensional interactions between target cells and substrate are important in DBP-induced endochondral osteogenesis. Reddi and Huggins examined the influence of matrix geometry on the rate and extent of bone formation in rats and found that demineralized powders of bone matrix (74–420 μm) had intense chemotactic attraction for fibroblasts that preceded chondrogenesis[9]. Both of these manipulations, i.e. production of regular crevices and reduction of bone to powders, increase cell–matrix interactions and the contact of target cells with the inducing substrate. Packing density of powders must also permit cellular invasion and contact with the surfaces of the particles. In other studies comparing tissue reactions to loose bone particles and particles that were compacted into a pellet, it was shown that compacted particles behave like a large block of bone with diminished cellular infiltration and reaction[10]. In order to increase contacts between cells and inductive particles of demineralized bone in cell culture, we developed a collagen/DBP sponge system that allowed cellular migration to particles of DBP supported in a collagen sponge[11]. We made those DBP/collagen sponges by the lyophilization of an admixture of DBP and collagen solution. Cells migrated into the sponges and attached to the particles of DBP, which were distributed through the collagen network. There was a limit to the density of DBP that could be contained in such a collagen sponge, however; sponges with a higher ratio of DBP to collagen were friable and unacceptable.

In this report, we describe a new 3-dimensional DBP/collagen composite sponge to assess the chondroblastic differentiation of human dermal fibroblasts. In this version, a packet of DBPs is contained in a pocket between two collagen layers. Collagen concentration and source, irradiation, and lyophilization conditions were varied to optimize cellular migration, biocompatibility and rigidity of the collagen sponge.

MATERIALS AND METHODS

Demineralized bone powder (DBP)

Bone particles were prepared from rat long bones (CD, Charles River Breeding Laboratory, Wilmington, MA) as described previously[12]. Briefly, cortical bone was harvested from femoral and tibial diaphyses. Connective tissue and marrow were removed and the bone reduced to small chips, approximately 5 mm in each dimension. The bone chips were washed vigorously in sterile water at 4°C overnight. The bone was extracted with absolute ethanol and subsequently was dehydrated with anhydrous ether. After air drying, the bones were fragmented in a liquid-nitrogen impacting mill (Spex Industries, Metuchen, NJ). Bone particles were sieved and collected as a fraction between 75 and 250 μm. Demineralized bone powder (DBP) was prepared by acid-extraction of the bone particles in 0.5 N HCl (50 ml per gram of bone powder) for 3 hours at room temperature. The bone powder was washed with batches of sterile distilled water by centrifugation at 10 000 × *g* for 10 min at 4°C until the pH of the supernatant became the same as the rinsing water (at least 500 ml per gram of bone particles). The powder was extracted with absolute ethanol and ether. Residual ether was evaporated under a chemical hood.

Guanidine-extracted DBP residue (ge-DBP)

A portion of the DBP was extracted with 4 M guanidine hydrochloride (30 ml per gram) at 4°C for 18 h in 50 mM Tris–HCl (pH 7.4) with protease inhibitors, 5 mM benzamidine, 0.1 M 6-aminohexanoic acid, 0.5 mM phenylmethylsulphonyl fluoride and 5 mM *N*-ethylmaleimide[13]. The residues were rinsed with sterile cold water (300 ml per gram of demineralized bone particles) and lyophilized overnight.

Mould for fabrication of collagen sponge

Medical grade Tygon™ tubing, S-50-HL (ID 3/8", OD 9/16", Norton, OH) was cut into 1 cm segments, washed in 7X™ detergent with sonication and autoclaved. One end of the segment was fixed to a polystyrene culture dish (Falcon, Franklin Lakes, NJ) with medical-grade silicon adhesive (Dow Corning, Midlang, MI). After overnight drying under aseptic conditions, these moulds were used for making collagen sponges.

Variations in the preparation of 3-dimensional collagen sponges

The following variables were evaluated for preparing 3-dimensional collagen sponges: source of collagen, concentration of collagen, lyophilization and ultraviolet light irradiation (*Table 1*).

Bovine pepsin-digested collagen solutions (0.1, 0.3, or 0.5% wt/vol, Cellagen™ PC-5, ICN Biomedicals, Costa Mesa, CA and VITROGEN 100™, Celtrix Laboratories, Palo Alto, CA) or bovine acid-soluble skin collagen (Verax, Lebanon, NH and ICN Biomedicals) were evaluated. These solutions were diluted with 0.05 M acetic acid or 0.012 N hydrochloric acid. For neutralization, one volume of 1 M HEPES (pH 7.4) and one volume of 1 M $NaHCO_3$ were added to 100 volumes of the collagen solution. A 120 μl volume of these collagen solutions was poured into the Tygon mould and frozen at −20°C. A moist piece of folded tissue paper (Kimwipes, Kimberly-Clark, Roswell, GA) was placed on some of these before the addition of a second aliquot (130 μl). Another piece of moist paper was placed on some of these before lyophilization. Some of the sponges were irradiated by UV light for 3 hours on each side in a laminar-flow safety cabinet.

Commercially obtained acid-soluble bovine dermal collagens and pepsin-digested bovine dermal collagen

Table 1 Variables in preparation of collagen sponges

Collagen type:	Bovine pepsin-digested (ICN or Celtrix) Bovine acid-soluble (Verax or ICN)
Collagen conc.:	0.1%, 0.3%, 0.5% (wt/vol)
Lyophilization	Without surface paper With surface paper
Terminal process:	Without UV With UV

were used to prepare sponges at 0.1, 0.3 and 0.5% (wt/vol) solutions. Sponges prepared from solutions containing 0.5% acid-soluble or 0.3 and 0.5% pepsin-digested bovine skin collagen were undamaged during handling or experimentation. On the other hand, 0.1 and 0.3% acid-soluble and the 0.1% pepsin-digested collagen sponges were too fragile to make useful sponges.

For demonstrating the surface properties of the collagen when formed according to these different processes, collagen sponges were sputter-coated with gold and were observed by scanning electron microscopy (AMRAY 1000A).

Mean pore width was estimated as $1/n \times \Sigma n\sqrt{sl}$, where s is the shortest, l is the longest width of each pore, and n is the sample number. These measurements were made on histological sections at ×200 magnification[14].

Fabrication of DBP/collagen composite sponges

Experience with different fabrication methods led to the method for preparing bilaminated devices with favourable physical properties and cellular interactions. This method comprised the following features: 0.5% pepsin-digested dermal collagen, UV-irradiated lattice geometry, surface porosity maintained by avoiding formation of a skin upon lyophilization, and creation of pocket to contain a mass of DBP between the two collagen layers of the composite sponge.

Having made the above evaluations in fabricating collagen sponges, the following method was developed for preparing composite DBP/collagen sponges to assess chondroinduction. Bovine pepsin-digested collagen (0.5% wt/vol, Cellagen™ PC-5, ICN Biomedicals, Costa Mesa, CA) was used for sponge fabrication. For neutralization of the solution, one volume of 1 M HEPES (pH 7.4) buffer and one volume of 1 M $NaHCO_3$ were added to 100 volumes of the collagen solution. A 120 μl volume of the collagen solution was poured into a mould and frozen at −20°C. A moist piece of folded tissue paper (Kimwipes) was placed on the surface of the frozen collagen as a spacer, allowing a small edge of the sponge to remain uncovered. One hundred and thirty μl of ice-cold collagen solution was layered over the spacer and the device was refrozen. Another piece of moist paper was placed over the frozen collagen to avoid the formation of a surface collagen skin upon lyophilization. After lyophilization at less than 10 μm Hg and −50°C for 18 h (LYPH-LOCK 4.5, LABCONCO, Kansas, MO), the surface paper was removed. The sponge within the mould was irradiated by UV light for 3 hours in a laminar-flow safety cabinet. The sponge was removed from the mould and the spacer was carefully removed from between the layers of the collagen sponge. The reverse side of the sponge was irradiated by UV light in the same manner.

Assembly of the culture device (*Figure 1*)

Tygon tubing with the same specification as that used for fabrication of the collagen sponges was cut into 1 cm segments. Conduits were made on the bottom for medium exchange. Medical grade silicon tubing (i.d. 7 mm, o.d. 10 mm; Iuchi, Tokyo) was cut into 1 mm thick rings for sealing and holding the sponge within the tubing. These parts were assembled in a laminar-flow safety cabinet. One silicon ring was inserted into the segment of Tygon tubing to hold the sponge for subsequent culture with cells. The bilaminate collagen sponge was placed on the silicon ring. An aliquot of 3 mg of DBP or ge-DBP was deposited between layers of the sponge with a dental amalgam carrier. The DBP/collagen composite sponge was sealed with another silicon ring (*Figure 1*).

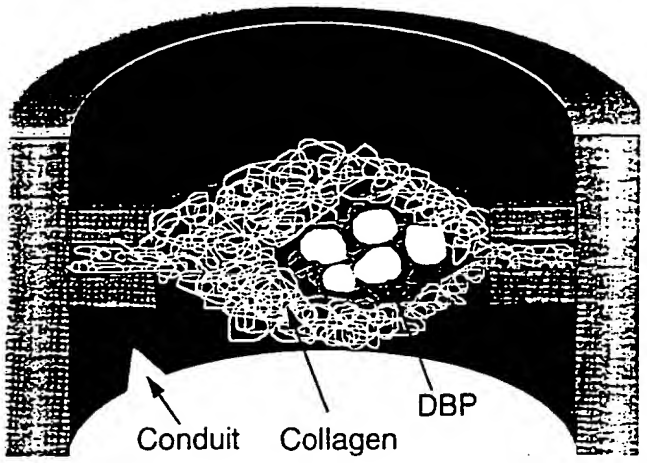

Figure 1 A DBP/collagen composite sponge held in tubing in which conduits facilitate medium exchange.

Cell culture

Human dermal fibroblasts (hDF) were cultured in Dulbecco's Modified Eagle Medium (D-MEM, GIBCO/BRL Laboratories, Gaithersburg, MD) with 10% fetal bovine serum (GIBCO/BRL Laboratories) and 1% penicillin/streptomycin (GIBCO/BRL Laboratories). Primary human dermal fibroblast cultures were prepared from minced explants of foreskin tissue of a 7-year-old patient. Foreskin was treated with 10% iodine–phosphate-buffered saline (PBS) for 2 min, and rinsed five times with PBS. The foreskin was minced into 1 mm^3 pieces and the pieces were transferred to 35 mm dishes (Falcon, Franklin Lakes, NJ). After overnight incubation of the explant tissue with 0.5 ml of medium, an additional 1.5 ml of the medium was added. Cultures at passages 12 through 18 were used for the experiments. One million cells in 50 μl were either deposited onto the sponge in the culture holder or injected into the sponge with a 25-gauge needle (Becton Dickinson, Rutherford, NJ) and 1 ml syringe (Becton Dickinson). The cultures were incubated at 37°C in 5% CO_2 in air. One hundred μl of medium was added 1 hour after seeding. After 3 hours, the cultures were transferred to 8 ml of medium and turned to the vertical position in six-well deep plates (Falcon) for efficient nutrient exchange. Duplicate cultures were harvested for histological analysis at 1 and 2 weeks after seeding. The medium was changed twice each week.

Animal study for validation of the system

Each sponge was rehydrated and rinsed three times with PBS. Duplicate DBP/collagen composite sponges

or collagen sponges were implanted subcutaneously in the ventral thoracic region in 28-day-old male rats (CD, Charles River Breeding Laboratory, Wilmington, MA) while anaesthetized with Metofane™ (Pitman Moore, Mundelein, IL). Eleven days later, specimens were harvested and were prepared for histological analysis[12].

Histological analysis

Specimens were fixed with 2% paraformaldehyde, 0.1 M cacodylate buffer (pH 7.4) at 4°C, rinsed with 0.1 M cacodylate buffer and embedded in glycol-methacrylate (JB-4™, Polyscience, Warrington, PA). Twenty-μm sections from the *in vitro* studies and 4-μm sections from the *in vivo* study were stained with 0.5% toluidine blue-O (Fisher Scientific, Fair Lawn, NJ) at pH 4.0.

RESULTS

This *in vitro* demineralized bone powder (DBP)/collagen composite sponge was designed to duplicate the geometry and density of a subcutaneous implant of DBP. The collagen for the DBP carrier was selected from different sources and concentrations of collagen solution were based upon their *in vitro* and *in vivo* compatibility, reproducibility and ease of handling. The fabrication technique was refined to allow cellular migration into the composite sponge.

After direct lyophilization of the collagens, a shiny, non-porous surface was observed on the sponges. In the set of laminates in which a piece of moist paper was overlaid on the frozen collagen before lyophilization, the paper was easily removed from the surface and the collagen surface was porous and showed no visible skin. Scanning electron microscopy showed that the surface without the paper overlay had a thin skin (*Figure 2a*). In contrast, the surface of the collagen that had been overlaid with moist paper had open pores (*Figure 2b*). Cells migrated into the porous sponges but not into the sponges with the skin.

For fabrication of a DBP/collagen composite sponge, a pocket was created by placing a paper spacer between the layers of collagen for packing the DBP. The mean pore width in the lower layer was approximately 120 μm and in the upper layer was approximately 200 μm (*Table 2*). This difference was not statistically significant. The presence or absence of spacers or covers during fabrication had little effect on the internal porosity of the collagen.

Sponges were fabricated with and without UV-irradiation. Non-irradiated sponges were transparent and fragile after rinsing in PBS. Examination of histological sections showed that the non-irradiated collagen gel had an amorphous appearance. Cells failed to migrate within the gel and remained on the surface of the collagen sponge even after 1 week of culture (*Figure 3a*). In contrast, UV-irradiated collagen sponges were firm and had a porous fibrillar structure that resembled a lattice. Cells had migrated into the sponge and were stretched within the collagen lattice (*Figure 3b*).

These results led to the method for preparing bilaminated devices with favourable handling properties and cellular interactions. This method comprised the following features: 0.5% pepsin-digested dermal collagen, UV-irradiated lattice geometry, surface porosity maintained by avoiding formation of a skin upon lyophilization, and creation of pocket to contain a mass of DBP between the two collagen layers of the composite sponge.

Cells were seeded for culture with two different procedures (deposited onto the sponge and injected

Figure 2 Scanning electron micrographs of the surface of collagen sponges. **a**, After lyophilization without paper, a skin was formed on the surface. **b**, After lyophilization with moist paper covering the collagen, a more porous surface was produced. White bars indicate 1000 μm.

Table 2 The pore size of collagen sponge lattice, prepared with and without spacer and/or paper cover. The pore size was estimated by measuring the longest (*l*) and shortest (*s*) width of each pore in cross-section and calculating the square root of the product $s \times l$ ($n = 20$)

Two layered fabrication	Lower layer		Upper layer	
	μm	±s.d.	μm	±s.d.
No spacer/cover	133.7	±34.1	203.3	±73.3
No spacer/no cover	127.3	±45.6	186.0	±61.6
Spacer/no cover	124.4	±30.6	195.7	±77.6
Spacer/cover	125.2	±30.1	208.4	±83.6

into the sponge). The sponges onto which cells were deposited showed migration and viability of cells throughout the sponges (*Figure 3b*). The fibroblasts readily migrated into the collagen sponge and partially covered the sponge surface by 7 days after seeding. In contrast, many pyknotic cells were seen in the DBP/collagen composite sponge 7 days after injection into the core. Therefore, for further experiments the cells were seeded onto rather than into experimental sponges.

The cells migrated into the mass of the DBP and were attached to individual particles of DBP (*Figure 4a*). Cells in the DBP/collagen composite sponge were surrounded by metachromatic matrix. This metachromatic shift with toluidine blue occurs with highly sulphated extracellular matrix components and is characteristic of cartilage and not of other connective tissues. *Figure 4a* shows granular deposits on the fine matrix filaments surrounding cells adjacent to the DBP. Cells within the collagen matrix at a distance from the particles of DBP were not associated with such a matrix.

In the inactivated ge-DBP/collagen composite sponges, cells migrated into the ge-DBP mass. Cells cultured with ge-DBP were not associated with metachromatic extracellular matrix (*Figure 4b*). Cellularity within the plain sponges appeared similar for the DBP/collagen or ge-DBP/collagen composites.

Sponges were implanted in subcutaneous sites in rats for assessment of compatibility and chondroinduction. Evaluation of sponges made of acid-soluble collagen 11 days after implantation in the rat subcutaneous site revealed an inflammatory reaction not provoked by pepsin-digested collagen sponges. DBP/collagen composite sponges induced chondrogenesis subcutaneously; this shows that the pepsin-digested collagen had no detrimental effect on the inductive activity of the DBP.

DISCUSSION

Three-dimensional bilaminated composite sponges of collagen and demineralized bone powder (DBP) were evaluated for use as an *in vitro* model for osteoinduction. A 3-dimensional device was developed that allows cellular migration and compatibility with the collagen component whilst permitting analysis of inductive activity of the DBP.

Rigidity of the collagen sponge is necessary to enclose the mass of DBP in a packing density like that which is effective *in vivo*. Non-UV-irradiated collagen sponges lacked the desired handling properties and did not permit cellular migration into the sponge. Phase-contrast microscopy showed that UV-irradiated and non-irradiated collagen sponges had distinctly different configuration. Non-irradiated collagen sponges had the appearance of an amorphous gel when polymerized at neutral pH and 37°C. Such a gel does not allow cellular migration into it[14]. By contrast, UV-irradiated sponges were comprised of a thick, distinct and porous collagen lattice. This lattice of collagen fibres allowed cell migration towards the

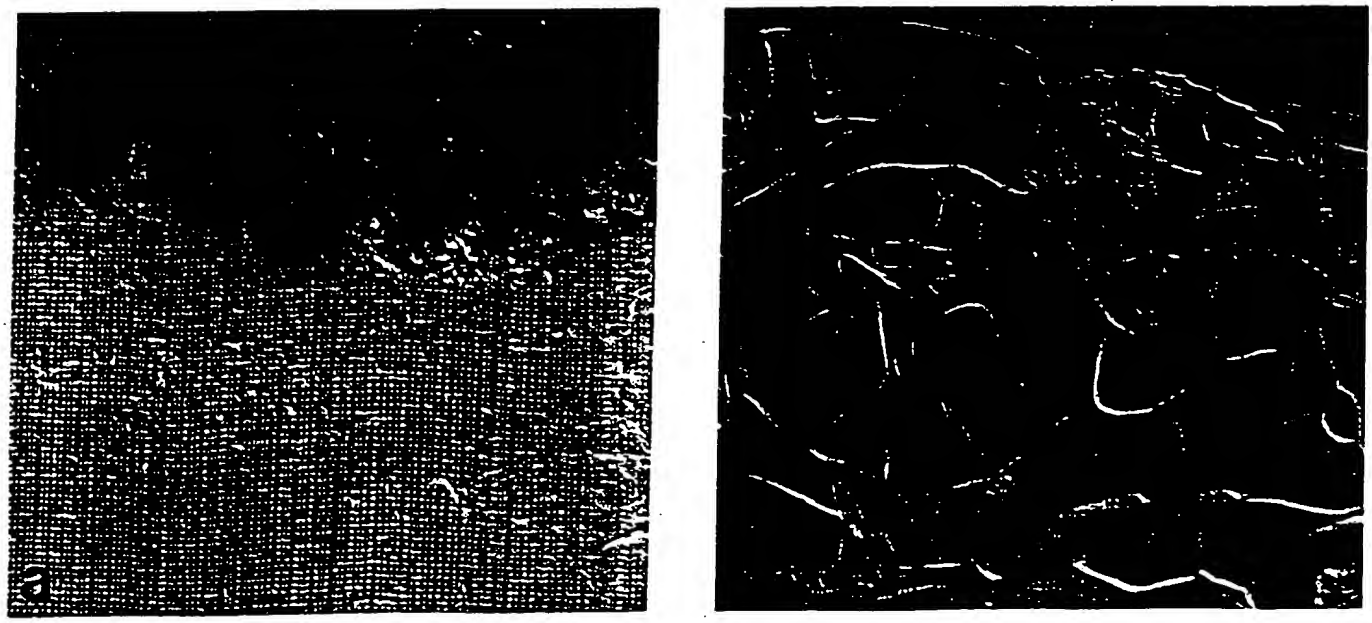

Figure 3 Photomicrographs of 20-μm-thick sections of collagen sponges 7 days after seeding with human dermal fibroblasts. a, In a collagen sponge not irradiated with UV light, the darkly stained area represents the mass of cells remaining on the surface of the amorphous collagen. b, In a collagen sponge irradiated with UV light, the arrows indicate parts of the collagen lattice. Human dermal fibroblasts are seen stretched within the collagen lattice. (Toluidine blue, ×125 original magnification.)

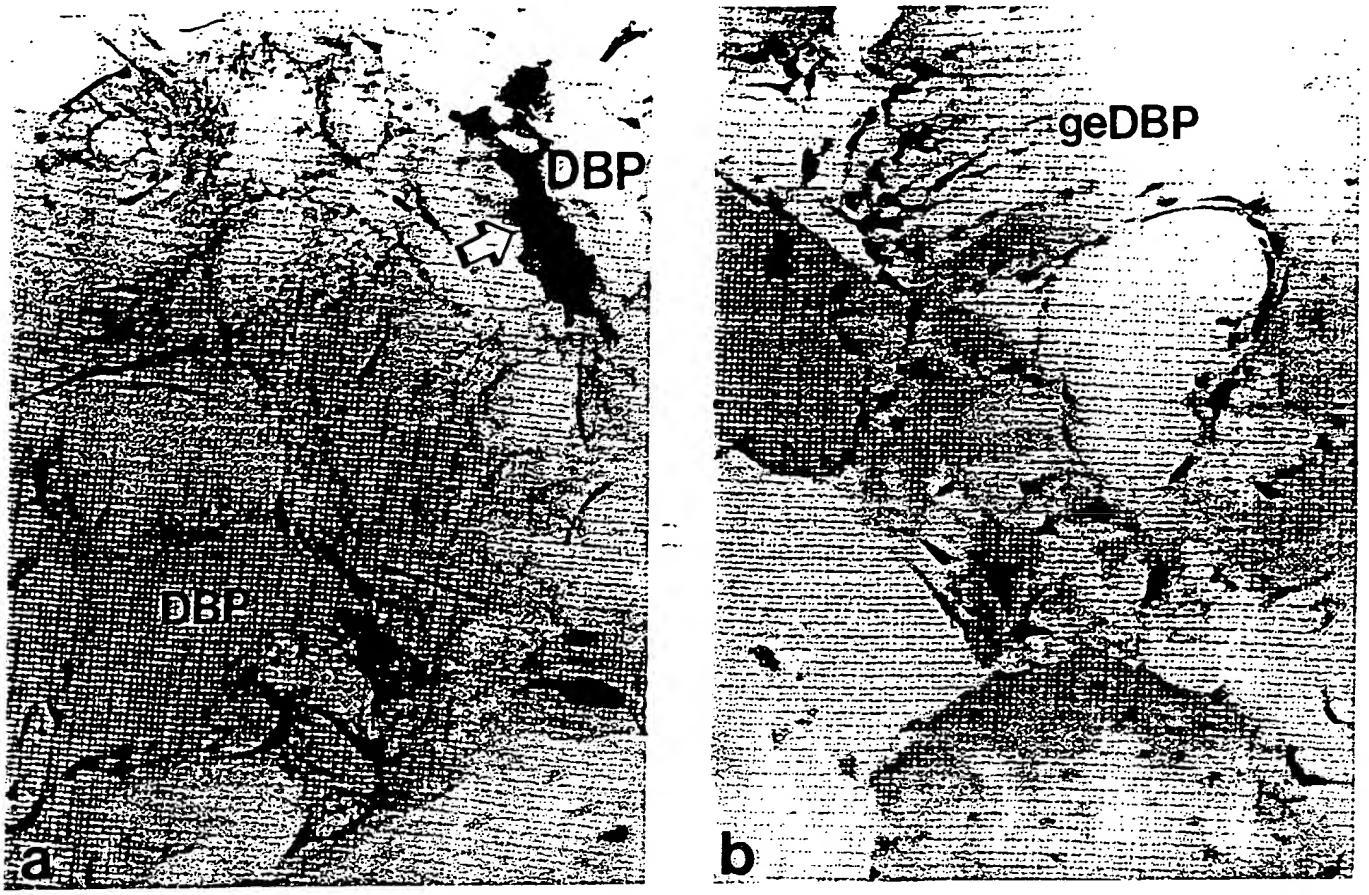

Figure 4 Photomicrographs of 20-μm-thick sections of sponges 7 days after seeding human dermal fibroblasts. **a**, Cells among particles of DBP are surrounded by granular deposits and filaments of extracellular matrix revealed by toluidine blue stain to be metachromatic (dark stain). Open arrow indicates positive stain in residual core of cartilage in DBP. **b**, Cells among particles of ge-DBP do not show this feature of cartilage extracellular matrix. (Toluidine blue, ×250 original magnification.)

enclosed DBP *in vitro* and *in vivo*. In contrast to hydrated collagen gels, cross-linked collagen has properties more useful as a tissue substitute. It is believed that UV-irradiation or chemical cross-linking stabilizes collagen fibrillar organization and decreases degradation and antigenicity[15]. UV-irradiation is one cross-linking procedure[16–18]. Others have used glutaraldehyde or other agents to covalently cross-link collagen for surgical implants[19], but these have not been extensively evaluated *in vitro*[20]. There are concerns, however, about *in vitro* cytoxicity with such material[21].

We tested several different concentrations and different sources of collagen. Acid-soluble collagen was excluded because inflammatory cells were seen with *in vivo* testing. The 0.1 and 0.3% collagen sponges were excluded from further evaluation because they lacked acceptable handling properties. 0.5% Pepsin-digested collagen made sponges with the required handling and compatibility properties.

Seeding of cells onto collagen composite sponges resulted in greater viability than when cells were injected into them. The fibroblasts were seen along and across collagen fibres and migrated into the sponges. Collagen fibres provide a favourable substrate for fibroblast attachment through adhesion molecules[22]. Other cell types have been shown to flourish on other prepared matrices. For example, MC3T3-E1, a clonal osteogenic cell line, produced abundant bone-like matrix when cultured within commercial 3-dimensional discs of processed tendon[23]. In addition, osteoblasts grew to high densities within fibres of poly-D-lysine-coated polyester fabric, where they deposited and mineralized bone matrix[24]. Hepatocytes maintained their phenotypic functions for more than 6 weeks *in vitro* when grown as a mass between two layers of hydrated collagen gel, but failed to do so when grown on a single layer of collagen[25]. That 'sandwich' culture system was designed to provide a scaffold to contain cells and their products and did not have an absolute requirement for collagen inasmuch as agarose gels could substitute for collagen[26]. Thus, although hydrated collagen gels can be useful for certain *in vitro* applications in which migration is not desirable and handling is not required, our results show the importance of UV treatment to obtain the porosity and mechanical properties required for our objectives.

A previous version of a DBP/collagen composite sponge was an admixture of particles of DBP distributed in a collagen matrix[11]. That system did not reproduce the packing density of DBP as it is deposited in animal models[8] or in clinical applications[12,27,28]. The use of a spacer between layers of collagen in the fabrication of the sponge creates a pocket that can accept particulate material at the desired packing density.

Cells seeded on top of such a sponge can migrate through the collagen lattice and interact with the

packet of DBP particles. When examined 1 week after seeding, the cells around and between these particles were surrounded by metachromatic extracellular matrix. Such metachromasia is characteristic of cartilage-specific extracellular matrix glycosaminoglycans[29]. Metachromatic matrix was not seen around cells in ge-DBP/collagen composite and the collagen sponges. Thus, specificity associated with osteoinductive DBP but not with inactivated ge-DBP was demonstrated.

This new *in vitro* DBP/collagen composite sponge mimics the 3-dimensional geometry and density of subcutaneous implants of DBP. This system will be valuable to help define (1) the mechanism of differentiation by osteoinductive materials and (2) the influence of other extracellular components and soluble factors on cartilage morphogenesis.

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